International Journal of Biological Macromolecules xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Development, characterization, antioxidant and hepatoprotective properties of poly(ϵ-caprolactone) nanoparticles loaded with a neuroprotective fraction of *Hypericum perforatum*

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ARTICLE INFO

Article history:
Received 20 June 2017
Received in revised form 4 October 2017
Accepted 16 October 2017
Available online xxx

Keywords:
Hypericum perforatum
Quercetin
Biapigenin
Nanoparticles
Anti-oxidant
Hepatoprotective
In vitro release

ABSTRACT

Quercetin and biapigenin are antioxidant and neuroprotective compounds present in *Hypericum perforatum*, with potential application in neurodegenerative diseases. Quercetin has shown to have excellent antioxidant activities. Biapigenin possesses a distinctive mechanism of action, preventing the onset of calcium deregulation and mitochondrial dysfunction. The main aim of this study was to establish a new delivery system encapsulating *H. perforatum* neuroprotective fraction, isolated from it,containing quercetin and biapigenin into poly(ϵ-caprolactone) (PCL) nanoparticles. It also aimed to study its hepatoprotective potential. Different formulation parameters were optimized, resulting in a stable formulation of polymer:compounds ratio 1:0.1 with a mean particle size of 185 nm, zeta potential around −20 mV and association efficiency close to 100%. Compounds *in vitro* release, under physiological conditions, showed an initial burst followed by a sustained release. The antioxidant functional properties of these compounds were not altered by encapsulation. PCL-loaded nanoparticles protected $HepG_2$ cells from intrinsic compound toxicity at high concentrations. Depending on the incubation regimen, quercetin-biapigenin PCL-loaded nanoparticles or free compounds were more effective in protecting $HepG_2$ cells against *tert*-butylhydroperoxide-induced toxicity. This is the first report of the encapsulation of a quercetin-biapigenin mixture in a polymeric matrix, specifically, in PCL, with synergic anti-oxidant and hepatoprotective effects.

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1. Introduction

The central nervous system (CNS) is known being particularly sensitive to oxidative stress, which can be described as an imbalance between generation and elimination of reactive oxygen species (ROS) and reactive nitrogen species (RNS).

By-products of cells' energy production, ROS and RNS, result from the cellular redox process. These species play a dual effect either as toxic and/or beneficial compounds. At low or moderate levels, ROS and RNS exert beneficial effects on cellular responses and immune function [1]. At high concentrations, a disturbance in the pro-oxidant/antioxidant balance in favor of the pro-oxidant state may occur, which can lead to cell damage. Oxidative damage is, therefore, associated with aging and the development of several diseases such as cardiovascular and neurodegenerative disorders, cancer and diabetes, resulting in the loss of membrane integrity, structural and functional changes in proteins and gene mutations.

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https://doi.org/10.1016/j.ijbiomac.2017.10.103
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ROS and RNS generated during the body's metabolic reactions can react with some cellular molecules, such as lipids, proteins and DNA, damaging them [1,2].

It is therefore important to preserve redox environment and mitochondrial function of the cell. This can be achieved by avoiding the causes of oxidative stress and strengthen the defenses with the usage of endogenous antioxidants and the intake exogenous antioxidants [3]. Besides their usage in the prevention of neurodegenerative diseases, antioxidants could also be relevant on its treatment, as a single compound or in supplementary combination with drugs targeting other pathogenic mechanisms [4].

H. perforatum has been used as a medicinal plant for centuries, for the treatment of external and internal disorders, such as minor burns, wounds, skin inflammation, nerve pain, anxiety and mild to moderately severe depression, competing for status as a standard antidepressant therapy and being a valid herbal alternative to synthetic antidepressants [5].

H. perforatum extracts and compounds also showed to have relevant antioxidant and neuroprotective properties [2,6–9]. Specifically, a purified fraction containing quercetin and biapigenin has been extensively studied regarding its antioxidant and neuroprotective properties [6–9]. Quercetin has been described to protect against several oxidative insults and several types of ROS [8],and is considered the most potent scavenger of ROS, RNS and peroxynitrite of the flavonoid family [10]. Its ability to interact with multiple cellular targets is likely the basis of its pharmacological activity [11]. *In vivo* quercetin activity remains controversial. Quercetin presents low aqueous solubility and instability in physiological medium [12], depending on temperature and pH, which limits its bioavailability, reduces permeability, and suffers extensive first pass metabolism before reaching the local of action [13]. Biapigenin is a less studied compound, possessing a distinctive mechanism of action, compared to quercetin. It was shown to prevent the onset of calcium deregulation and mitochondrial dysfunction, affecting mitochondrial bioenergetics [6,8,9]. Antioxidant activities of biapigenin have also been reported through its lipid peroxidation inhibition potential [6,7].

One alternative to circumvent bioavailability problems is to entrap or adsorb the compounds into nanoparticles. The advantages of using nanoparticles for drug delivery result from their small size, which allows them to penetrate within small capillaries and be taken up within the cells, with efficient drug accumulation at the target sites in the body. It also allows sustained drug release within the target site over a period of days or even weeks after administration. [14]. Nanosystems employed for CNS targeted drug delivery include polymeric nanoparticles that compared with other colloidal carriers, are a relevant alternative. PCL is a semi-crystalline hydrophobic polymer, degraded by hydrolysis of its ester linkages under physiological conditions. Its high permeability to many drugs and a lack of toxicity has made PCL and its derivates well suited for drug delivery [15].

Liver is essential for survival because of its role in the coordination of the body's metabolism, including glucose homeostasis, xenobiotic metabolism and detoxification. All organs are vulnerable to oxidative stress. However, because of the high metabolic activity of hepatocytes, liver is highly susceptible to oxidative stress and a major target of toxic substances. Biotransformation of certain xenobiotics can also produce short-lived, unstable, highly reactive chemical species, such as ROS and RNS that can interact with functional biomolecules and lead to adverse effects, resulting in inflammatory and fibrotic liver disorders [16].

Human hepatocellular carcinoma cells ($HepG_2$) is a widely used human *in vitro* model for hepatic toxicity studies, including antioxidants that can be assayed with minor variations [16]. $HepG_2$ cells retain many of the specialized functions characteristic of normal human hepatocytes, including the synthesis and secretion of plasma proteins, phase I, phase II and antioxidant enzymes, being good tool to study hepatic toxicology, cytoprotective, and antigenotoxic effects of compounds [17].

The main aim of this study was to establish a nanoparticulate system for the use of a neuroprotective fraction of *H. perforatum* containing quercetin and biapigenin, using the synthetic polymer PCL, by solvent displacement technique. The physical and morphological parameters of the nanoparticles were investigated, as well as its *in vitro* release profile. Furthermore, the antioxidant activities of the compounds and of its nanoparticles were determined. The potential protective effect of quercetin-biapigenin and its PCL-loaded nanoparticles against *t*-BOOH-induced oxidative stress in $HepG_2$ cells was also studied.

2. Material and methods

2.1. Materials

Dulbecco's Modified Eagle Medium (DMEM), antibiotic-antimycotic solution, glutamine, trypsin-ethylenedi aminetetraacetic acid (EDTA) solution, 3-(4,5-dimethy lthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), PCL, Pluronic® F-68, DPPH, nitroblue tetrazolium (NBT), reduced form of nicotinamide adenine dinucleotide (NADH), N-phenylmethazonium methosulfate (PMS), quercetin, biapigenin, curcumin, ferrozine, trichloroacetic acid and tiobarbituric acid were purchased from Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA). Diethyl ether and acetone were purchased from Merck (Germany). Sephadex LH-20 and polyamide CC6 acquired were from GE Healthcare Life Sciences (UK). L(+)-Ascorbic acid and pyruvate were purchased from Panreac(Barcelona, Spain). EDTA was purchased from VWR (Portugal). Fetal Bovine Serum (FBS) was purchased from Biochrom KG (Berlin, Germany). All other reagents used were for analytical grade.

2.2. Methods

2.2.1. *Isolation of a neuroprotective* Hypericum perforatum *fraction containing quercetin and biapigenin*

H. perforatum plants (aerial portion) were collected in the region of Braga, North of Portugal, and the biomass was freeze-dried on the same day, in a Labconco lyophilizer at 0.01 mBar for 4 days, with a condenser surface temperature of −90 °C.

A methanolic extract was prepared by macerating plant biomass (100 g dwb/L) with a methanol-water solution (80:20, V/V), for 4 days, at room temperature and in darkness. The mixture was filtered through a paper filter (Whatman, No. 1) and the resulting methanolic extract was used for further processing. The acidified extract (pH 3) was fractionated by liquid–liquid extraction with diethyl ether and the resulting fraction concentrated, under reduced pressure, at 35 °C, in darkness. This ether fraction was further subjected to Sephadex LH-20 column chromatography, eluted with methanol. A selected fraction containing quercetin and biapigenin was collected, concentrated, and purified through Polyamide CC6 column. Compounds identification was performed by HPLC-DAD as previously described [18]. Chromatograms were recorded at 260, 350 and 590 nm, and quercetin and biapigenin were quantified (350 nm) by the external method, using pure commercial standards. This fraction is composed solely by quercetin and biapigenin and will be referred wherein as quercetin-biapigenin.

2.2.2. *Production of quercetin-biapigenin poly-(ϵ-caprolactone) (PCL) nanoparticles*

Different formulations of quercetin-biapigenin loaded PCL nanoparticles (PCL: quercetin-biapigenin ratio of 1:0.1; 1:0.2 and 1:0.5) were prepared by nanoprecipitation, as described elsewhere [19]. Briefly, the corresponding mass of quercetin-biapigenin and

Please cite this article in press as: A.I. Oliveira, et al., Int. J. Biol. Macromol. (2017), https://doi.org/10.1016/j.ijbiomac.2017.10.103

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